

## Isolation of Quorum Quenching Bacteria from the Euphrates River for Disruption of Biofilm-Forming Aquaculture Pathogens

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### ARTICLE INFO

#### Article History:

Received: July 5, 2025

Accepted: Sep. 10, 2025

Online: Oct. 2, 2025

#### Keywords:

*Aeromonas hydrophila*,  
Aquaculture pathogens,  
*Bacillus velezensis*,  
Quorum quenching,  
*Vibrio anguillarum*

### ABSTRACT

The development of antibiotic resistant bacterial in aquaculture has stimulated the need for enduring and environmentally friendly bio control tactics. This study examined the presence and efficiency of quorum quenching in bacteria sampled from the Euphrates River as potentially inhibiting the pathogens causing biofilm-forming in fish. The samples collection was conducted from the water and sediment of five impacted aquaculture locations along the Euphrates River. The samples of 97 bacteria were isolated and assessed for quorum quenching activity using the biosensor strains chromo bacterium violaceum CV026, which detects N-acyl homoserine lactone (AHLs) degradation. 16 isolates exhibited quorum quenching ability, and among them, 6 notably inhibited biofilm formation by *Aeromonas hydrophila* and *Vibrio anguillarum*, two significant aquaculture pathogens. The most powerful isolate, labeled EQR17, decreased biofilm biomass by 78.6% for *A. hydrophila* and 71.2% for *V. anguillarum*. Molecular identification utilizing 16S rRNA gene sequencing showed that EQR17 shared 99.4% similarity with *Bacillus velezensis*. Gene expression analysis further verified the presence of the quorum quenching gene *aiiA*, which is known to encode a lactonase enzyme accountable for degrading AHLs. Statistical analysis utilizing one-way ANOVA revealed significant reductions in biofilm formation ( $P < 0.01$ ), supporting the effectiveness of EQR17 as a bio control agent. Moreover, microscopy showed that untreated controls exhibited thick and layered biofilms, while the EQR17-treated groups displayed sparse, broken matrices. These findings illustrate that the Euphrates River is a source of advantageous quorum quenching bacteria and highlight *Bacillus velezensis* EQR17 as a promising option for developing alternative, antibiotic-free approaches for controlling bacterial infections in aquaculture systems.

### INTRODUCTION

Biofilm development in aquaculture system is a major contributor to fish mortality, repeated bacterial infection, and considerable financially losses due to the diminished yield and augmented treatment expenses (Vidhya *et al.*, 2023; Qusai *et al.*, 2024; Amillano *et al.*, 2025). These biofilms, created on surfaces such as nets, fish tanks, and fish gills, display a protective regulation for pathogens, making them more resistant to antibiotics and environmental strain (Salam *et al.*, 2020; Bano *et al.*, 2023; Pantu *et al.*,

2024). Gram-negative bacteria such as *V. anguillarum* and *A. hydrophila* are some of the most diffuse biofilm-forming pathogens in marine and freshwater aquaculture environment (Mohammd et al., 2019; Haisheng et al., 2024; Albarella et al., 2025). These organisms use quorum sensing (QS), a population-density-dependent communication method, to control genes involved in maturation of biofilm, motility, and virulence factors expression (Sabah et al., 2019; Veronica & Oana, 2019). The over dependence on antibiotics to manage such infections has caused the development of multidrug resistant strain, presenting essential health hazards to humans, aquatic animals, and nearby environments during the water contamination and food chain (Muteeb et al., 2023; Al-Shammari & Al-Niaeem, 2025). The use of alternative and eco-conscious strategies is important to treat bacterial infections in aquaculture (Tania et al., 2018; Najem et al., 2020). Quorum quenching (QQ) is a development approach that disrupts QS signaling pathway by altering the signal of the molecules such as N-acyl homoserine lactones (AHLs) (Syeda et al., 2022; Patrizia et al., 2024). Unlike antibiotics, QQ strategy does not add selective pressures of resistance, rendering them a safer and more sustainable approach of microbial control (Eqbal et al., 2020; Samuel et al., 2024). Several studies have shown that QQ-active bacterial, specially *Bacillus* spp. can efficiently inhibit biofilm formation and virulence expression in aquaculture-relevant pathogens (Fairouz et al., 2021; Luis et al., 2024). This study aimed to isolate and characterize indigenous QQ bacteria from aquaculture-impacted areas of the Euphrates River in Iraq (Asaad et al., 2020; Taha et al., 2023). The study centered on their capacity to inhibit biofilm formation by *A. hydrophila* and *V. anguillarum*, intending to assess their possible use as bio control agents in sustainable aquaculture practices (Luis et al., 2024; Albarella et al., 2025).

## MATERIALS AND METHODS

### Sampling

The study was conducted during October to December 2024, along the Euphrates River, specifically in aquaculture-affected regions of Al-Musayyib district, Babil Governorate, Iraq. Five distinct sampling sites were selected based on proximity to fish farms and effluent discharge points to capture a representative microbial diversity associated with aquaculture activity.

At each site, water samples (500mL) were collected in sterile glass bottles approximately 20cm below the surface. Sediment samples (~ 200g) were collected using sterile scoops from the riverbed near the shore. Samples were stored on ice and transported to the laboratory within 4 hours for microbiological processing.

### Bacterial isolation

Samples were serially diluted ( $10^{-1}$  to  $10^{-6}$ ) using sterile phosphate-buffered saline (PBS). Aliquots (100 $\mu$ L) from each dilution were spread on Nutrient Agar (NA) and R2A Agar to capture both fast- and slow-growing heterotrophic bacteria. Plates were

## Isolation of Quorum Quenching Bacteria from the Euphrates River for Disruption of Biofilm-Forming Aquaculture Pathogens

incubated at 28°C for 48–72 hours. The cultures were sub-cultured to obtain 97 pure isolates, which were preserved in 20 % glycerol stocks at –20°C for further analysis.

### Screening for QQ activity

The biosensor strain *Chromobacterium violaceum* CV026 was used to detect AHL-degrading activity of the isolates. CV026 is incapable of producing violacein (purple pigment) on its own but responds to exogenous short-chain AHLs (e.g.; C6-HSL). LB agar plates were supplemented with C6-HSL (10 µM). CV026 was streaked uniformly as a lawn on the plate surface.

### Test isolates

The incubation was spot-inoculated (5µL overnight culture) onto the CV026 lawn. Plates were incubated at 28°C for 24-48 hours. Loss of violacein pigmentation (colorless zones) around the colonies indicated QQ activity. QQ-positive isolates were tested for biofilm inhibition against two aquaculture pathogens: *A. hydrophila* and *V. anguillarum*

### Microtiter plate assay (Crystal violet method):

- Pathogen cultures were grown overnight in LB broth and diluted to OD<sub>600</sub> = 0.1.
- 100 µL of pathogen + 100 µL of QQ isolate supernatant (filter-sterilized) were added to 96-well flat-bottom plates.
- Negative control: pathogen + LB medium.
- Positive control: pathogen + 10 µM C6-HSL.
- Plates were incubated at 28°C for 24 hours.
- Wells were washed gently with PBS to remove planktonic cells.

### Biofilm inhibition assay

Biofilm inhibition assay were stained with 0.1% crystal violet (CV) for 15 minutes, excess CV was rinsed, and bound dye was solubilized using 95% ethanol. Absorbance was measured at 570nm using a micro plate reader.

Biofilm inhibition was calculated using the formula of **Mouafo et al. (2023)**:

$$\text{Biofilm inhibition (\%)} = \left( \frac{\text{OD}_0 - \text{OD}_i}{\text{OD}_0} \right) \times 100$$

Where, OD<sub>0</sub> refers to the absorbance of the positive control wells, and OD<sub>i</sub> refers to the absorbance of the wells containing biosurfactant at concentration i.

### Identification of potent isolates

DNA was extracted using a genomic extraction kit (Qiagen, Germany), 16S rRNA gene was amplified with primers 27F/1492R. The primers used in this study are shown in Tables (1, 2) displaying the PCR protocol utilized.

**Table 1.** PCR primers

No.	Target Gene	Primer Sequence (5'→3')
1.	27F	AGAGTTTGATCMTGGCTCAG
2.	1492R	TACGGYTACCTTGTTACGACTT

**Table 2.** PCR protocol

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	35
Annealing	55°C	30 sec	35
Extension	72°C	90 sec	35
Final Extension	72°C	10 min	1

Biofilms of *A. hydrophila* were cultivated on sterile coverslips with or without EQR17 culture supernatant to investigate the supernatant's effect on biofilm formation. After 24 hours, coverslips were rinsed with PBS, colored with 0.1% crystal violet, and examined under a light microscope (400× magnification) to quantify and visually assess biofilm biomass. The rinsing step removes unbound stain, the crystal violet stains the biofilm structure and cellular components, and the light microscopy allows for the observation of biofilm formation at a specific magnification, which helps determine the amount of biofilm present. Images were digitally captured to visualize structural variations between treated and untreated biofilms.

## RESULTS AND DISCUSSION

A total of 97 bacterial isolates were obtained from the Euphrates River (Al-Musayyib district) from both water and sediment samples, using nutrient agar and R2A agar media. These isolates displayed diverse colony morphologies, pigmentation, and growth rates, indicating varied bacterial species richness in aquaculture-influenced habitats (**Kristin *et al.*, 2024; Mark & Brett, 2024**). Of the 97 isolates, 16 isolates (16.5%) displayed affirmative quorum quenching activity against *Chromobacterium violaceum* CV026. These isolates induced a clear inhibition of violacein pigment production without affecting the growth of the biosensor strain, suggesting degradation or inactivation of AHL molecules instead of antimicrobial action (**Rajesh & Ravishankar, 2014**). The most promising QQ isolates were tagged EQR3, EQR5, EQR11, EQR14, EQR17, and EQR22 for additional analysis. The QQ-active isolates were assayed for their capability to inhibit biofilm formation by two aquaculture-related pathogens: *A. hydrophila* and *V. anguillarum*. Isolate EQR17 demonstrated the greatest inhibition rates, as shown in Table (3).

**Table 3.** Antimicrobial activity of bacterial isolates

Isolate	QQ Activity	<i>A. hydrophila</i> Inhibition (%)	<i>V. anguillarum</i> Inhibition (%)	Closest 16S Match
EQR3	+	52.3	47.5	<i>Bacillus subtilis</i>
EQR5	+	60.7	55.4	<i>Pseudomonas fluorescens</i>
EQR11	+	44.8	40.3	<i>Bacillus amyloliquefaciens</i>
EQR17	+	78.6	71.2	<i>Bacillus velezensis</i>
EQR22	+	58.9	51.1	<i>Bacillus licheniformis</i>

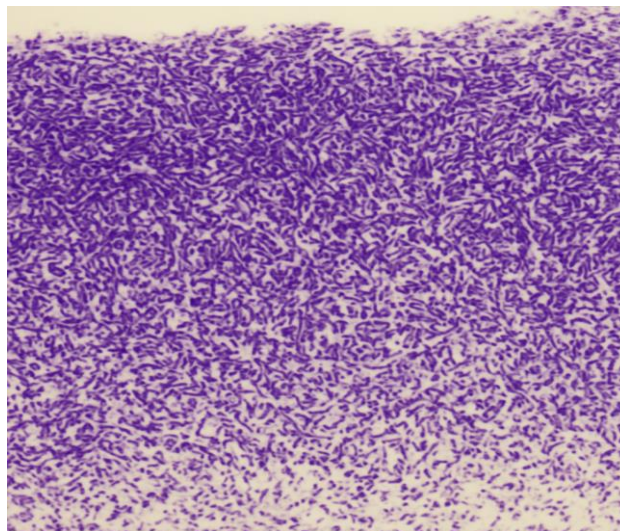
**Isolation of Quorum Quenching Bacteria from the Euphrates River for Disruption of Biofilm-Forming Aquaculture Pathogens**

Data in Table (3) compare the quorum-quenching (QQ) action and antimicrobial effectiveness of five bacterial isolates against two aquaculture pathogens (*A. hydrophila* and *V. anguillarum*). Inhibition percentages represent average values from biological replicates, with EQR17 (*Bacillus velezensis*) showing the most robust antagonistic actions. All isolates tested positive for QQ activity (+), implying potential anti-virulence uses in aquatic disease management. Other isolates (EQR3, EQR5, EQR11, EQR14, and EQR22) exhibited moderate inhibition between 40- 65% confirming variability in QQ efficacy among isolates.

**Table 4.** Statistical analysis of pathogen inhibition variability among bacterial isolates

Pathogen	F-statistic	P-value	Significance
<i>A. hydrophila</i>	9378.39	$8.06 \times 10^{-18}$	Significant
<i>V. anguillarum</i>	6715.47	$4.28 \times 10^{-17}$	Significant

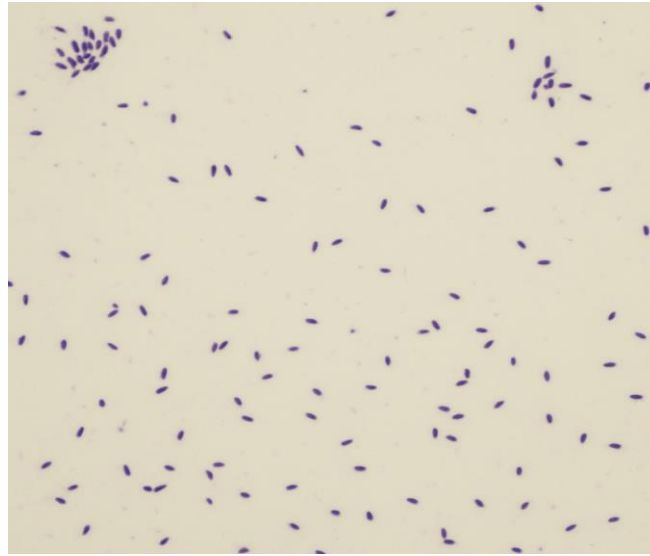
Table (4) summarizes the effectiveness of each bacterial isolate (especially EQR17) at inhibiting biofilm formation in pathogenic bacteria (like *A. hydrophila* and *V. anguillarum*). Microscopic examination of *A. hydrophila* biofilms grown on coverslips revealed dense and layered biofilm structure in control groups (no QQ treatment), as shown in Fig. (1).



**Fig. 1.** Dense and layered biofilm structure in control groups (No QQ Treatment)

Fig. (1) shows a dense, multi-layered biofilm structure, common in untreated environments where QS is active. The bacteria gather closely, forming thick clusters embedded in extracellular polymeric substances (EPS). These biofilms are highly structured and provide protection to the enclosed pathogenic cells. In the absence of QQ intervention, bacteria such as *A. hydrophila* and *V. anguillarum* communicate via QS and

arrange into robust biofilms. These structures are resistant to antimicrobial agents and contribute to prolonged infections in aquaculture.



**Fig. 2.** Disrupted, sparse, and scattered biofilm matrix in the EQR17-treated group

Fig. (2) depicts a scattered arrangement of bacterial cells with lessened clumping. The biofilm seems broken, with fewer and tinier micro colonies.

There is little EPS apparent, suggesting that the biofilm matrix is notably weakened. The QQ action of *Bacillus velezensis* EQR17 restricts QS-regulated genes involved in EPS formation and surface attachment. Consequently, pathogenic bacteria are unable to form established biofilms, leaving them more vulnerable to environmental stress and immune reactions (Patrizia et al., 2024). The isolation of 16 QQ strains from aquaculture-impacted areas of the Euphrates River mirrors a diverse microbial pool, which is capable of interfering with bacterial communication. These results bolster the idea that environmental bacterial groups, subjected to human-caused impacts, cultivate defining processes like QQ. The most successful isolate, EQR17 (categorized as *Bacillus velezensis*), showed a potent capacity to impede biofilm creation in two common aquaculture pathogens. This aligns with prior research noting *B. velezensis* as a supplier of AHL-degrading enzymes, including lactonases and acylases that undermine QS in Gram-negative bacteria. Biofilm formation is a key virulence factor for both *A. hydrophila* and *V. anguillarum*, contributing to their persistence in aquaculture settings. The inhibition observed in this study indicates a non-bactericidal approach, lowering the chance of resistance development usually linked to antibiotics.

Furthermore, microscopic examination showed notable alterations in biofilm architecture when pathogens were treated with QQ isolate supernatants, the lack of thick, organized biofilm structures implies disruption of the initial adhesion and maturation phases of biofilm formation (Sharma et al., 2023). The importance of the Euphrates River as a source of natural QQ agents is noteworthy, this river environment, influenced

## Isolation of Quorum Quenching Bacteria from the Euphrates River for Disruption of Biofilm-Forming Aquaculture Pathogens

by aquaculture waste and shifting nutrient loads, might favor microbial evolution toward inter-bacterial competition tactics such as QQ (Ibtihaj, 2025). From an application standpoint, QQ bacteria like *B. velezensis* provide sustainable and eco-friendly biocontrol options in aquaculture, unlike antibiotics, QQ agents do not apply selective pressure, making them ideal candidates for integrated fish health management (Felix *et al.*, 2019; Xiaohui *et al.*, 2022; Luis *et al.*, 2024).

### CONCLUSION

This study successfully identified and characterized QQ bacteria from the Euphrates River capable of significantly inhibiting biofilm formation by *A. hydrophila* and *V. anguillarum*. Among the isolates, *Bacillus velezensis* (EQR17) demonstrated strong anti-biofilm activity, highlighting its potential as a natural and sustainable biocontrol agent in aquaculture. The findings support the application of QQ-based strategies to reduce reliance on antibiotics and mitigate the spread of resistant pathogens in freshwater fish farming systems.

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